Effect of encapsulation on survival of *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083 under temperature stress and storage in food matrix

FOR THE PARTIAL FULFILLMENT OF THE MASTER
OF SCIENCE DEGREE IN LIFE SCIENCE

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Date: 11th May, 2015
Place: Rourkela **RINA YADAV**

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CERTIFICATE

This is to certify that the thesis entitled **"Effect of encapsulation on survival of *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083 under temperature stress and storage in food matrix"** which is being submitted by **Ms. Rina Yadav,** Roll No. **413ls2055**, for the degree of master of science in Life Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institute for the award of any degree or diploma.

R. Jayabalan.

DECLARATION

I do hereby declare that the Project report entitled **"Effect of encapsulation on survival of *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083 under temperature stress and storage in food matrix"** submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfillment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of **Dr. Rasu Jayabalan**, Assistant Professor, Department of life Science, NIT, Rourkela.

Date: 11th May 2015

Place: Rourkela

RINA YADAV
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LIST OF ABBREVIATIONS

G	**Gram**
mg	**milligram**
L	**litre**
ml	**millilitre**
◦	**degree**
C	**centigrade**
H	**hour**
m	**min**
%	**percentage**
TSS	**Total soluble solid**
M	**molar**
mM	**mill molar**
CFU	**Colony forming unit**

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Abstract

The objective of this study is to examine the temperature tolerance of bacterial cells and their viability after storage in the food matrix. Though probiotics are sensitive to temperature, encapsulation was used to protect the cells from various stress tolerance like temperature. For the present study *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083 were selected. The bacterial cells were exposed at different temperature for different time intervals in water or oil bath, then their survivability was checked by spread plate method using MRS agar. The CFU/ml was calculated for *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083 after incubation for 48 hrs at 37^0C. It was observed that *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083 could tolerate up to 90^0C for 1 min and 100^0C for 30 sec, respectively as free cells. *Lactobacillus plantarum* NCIM 2083 was encapsulated using alginate (3 %) and the tolerance of encapsulated cells was studied. It was found that encapsulation could enhance the temperature tolerance of *Lactobacillus plantarum* NCIM 2083 up to 130^0C for 2 min. *Lactobacillus plantarum* NCIM 2083 as free cells was incorporated in the orange peel jelly and their viability was checked. Synbiotic microcapsules were are stored in health mix (Horlicks) for two weeks and the viability of cells in food matrix was checked after 2 weeks.

Key words: Viability, Synbiotic, Encapsulation, Prebiotic

1. Introduction

The probiotics are the living microbes when consumed in adequate amount confer various health benefit effect like improving microbial flora, reducing cholesterol, diarrhoea and constipation, etc. The intake of certain species of probiotic microorganisms is advantageous for reducing the duration and severity of diarrhoea signs. The food in the diet containing constructive bacteria can be used as a strategy for preventing diarrhoea. In order to have the beneficial effect the amount of probiotic in the ingested food should be at least 10^7 CFU/g. The most common bacteria commercial available are of the genera *Lactobacillus* spp. These bacteria are typically saccharolytic (capable of metabolizing sugars), gram-positive, rod shaped and reside in the large bowel (Cook et al, 2012). The key benchmarks involved in the selection of probiotic microorganisms include the origin and biosafety of the strains.

The viability of the cells is crucial because they need to be viable in order to have the above mentioned effects. Exposure to various factors like pH, high temperature and high osmotic pressure etc. which may affect the survival of probiotics in foods during processing and storage hence it is important to examine these factors through all steps from adding to the foods to delivery in the digestive system. The main challenges for the producers in the present market is the product manufacturing under unfavourable conditions (heating). Heating process is applied during production of many food products as it is sometimes an integral part of the production process, for example bread making. Microencapsulation is the method of applying a covering to sensitive probiotic to protect them from their external environment (Capela et al, 2007) and the probiotic cells are retained within an encapsulating matrix. Microencapsulation of probiotics has been examined for its ability to increase the viability of probiotic in food products.

2. Review of literature

2.1 Probiotic viability in stress environment

Probiotics are the gentle organisms that require appropriate treatment to maintain maximum activity. Although Probiotic strain vary in their sensitivity to heat but most of studies found that bacterial organism lose their viability overtime at room temperature. Probiotics encapsulation using alginate, alginate-chitosan by emulsification/internal gelation technique can reduce their stress by an extra covering.

2.2 The effect of temperature on the survival of free probiotics

During heating process temperatures above 90^0C may kill free probiotic cells, exposure of probiotic cells at 90^0C for 30 min, resulted in decrease in two cultures of free living cells ranging from 3.6 to 5 log reduction depending on their strain. Thus, D_{value} (the required time for reducing one log) could be estimated as 6-10 min. Accordingly, it is suggested, that 3 log losses occurs during pasteurization with low temperature (63^0C) for 30 min. These decreases in survival of probiotics are very high explaining the addition of the cultures after pasteurization Champagne, (2009) also 5.8-6.7 log reduction has been observed in orange jelly

inoculated with four probiotic strains (*L. fermentum, L. plantarum, L. acidophilus, L. bulgaricus*) stored at room temperature for 1 week. Two strain *L. fermentum, L. bulgaricus* are sensitive and *L. plantarum* and *L. acidophilus* are showing resistant (least loss).

2.3 The effect of temperature on the viability of microencapsulated probiotics

Microencapsulation means surrounding small particles of solid, liquid droplets and gas in a cover. It is a new and effective technique applied in food industries for protecting probiotic cells in foods during passage through the gastrointestinal tract and may be used in the development of functional food products. The main purpose of this technique is to protect the cells against unfavourable conditions, keeping them alive and metabolically active in the gut.

Microencapsulating matter is dispersed in the matrix and is used as shell or cover. Food polymers such as alginate, starch, mushroom etc., may be used in this process. Alginate is the most commonly used biopolymer for microencapsulating because of being non-toxic, easy, and inexpensive (Ding and Shah, 2007). The resistance of four probiotic strains as free and encapsulated using alginate with respect to temperature (120^0C for maximum, 3 min). The results revealed that the encapsulated bacteria showed more survival and less loss. Therefore, it is recommended that the encapsulated bacteria are protected temporarily through reducing heat transfer into the cell. Increasing the concentration of alginate also resulted in improved survival of encapsulated bacteria. Less diffusion of water into the matrix of 3% alginate during heating seems to be the cause of increased survival of the bacteria. Applying other material along with alginate such as starch for microencapsulating may increase the viability and survival of probiotics.

3. Aims and Objectives

i)To examine the temperature tolerance of *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083.

ii)To study the effect of encapsulation of heat tolerance of *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083.

iii)To check the survivability of *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083 during storage in orange peel jelly as free cells.

iv)To check the survivability of *Lactobacillus plantarum* NCIM 2083 and *Lactobacillus acidophilus* NCIM 2660 in synbiotic microcapsules during storage in health mix.

4. Materials and Methods

4.1 Microorganisms

L. fermentum NCIM 2156 and *L. plantarum* NCIM 2083 cultures were maintained and sub-cultured in 500 ml MRS broth by inoculation of 100 µL culture from glycerol stock maintained in laboratory and incubated at 37^0C for 24 hrs. Cells were harvested by centrifugation at 7000

rpm for 20 min at 25^0C. Supernatant was discarded and pellets were suspended in 20 ml saline. The final cell concentration was adjusted to 1.3 x 10^{10}CFU/ml and 5.8 x 10^{10}CFU/ml for *L. fermentum and L. plantarum,* respectively. Colony forming units was calculated by using the following formula.

CFU/ml = Number of colonies x dilution factor/volume of culture plate

4.2 Microencapsulation

L. fermentum NCIM 2156 and *L. plantarum* NCIM 2083 were encapsulated in sodium alginate matrix. Sodium alginate solution (3%) and calcium chloride solution (0.5M) was prepared, sterilized by autoclaving (120^0C for 15 min) and cooled to 38^0C -40^0C. Sodium alginate solution (20ml) was added to the calcium chloride solution (40ml) in a drop wise manner with the help of syringe in order to make the beads. The beads were separated by filtration using Whatmann filter paper and was transferred to sterile falcons and stored in refrigerator.

4.3 Temperature tolerance of free cells

1 ml culture of *L. fermentum* NCIM 2156 and *L. plantarum* NCIM 2083 were transferred to each test tubes containing 9 ml distilled water. These test tubes were exposed to different temperatures (60^0C, 70^0C, 80^0C, and 90^0C) for different time intervals (1 min, 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min) in water bath. The cells were spreaded on MRS agar by spread plating method and viability of cells was examined after incubation at 37^0C for 48 hrs.

4.4 Temperature tolerance of encapsulated bacterial cells

Tolerance of encapsulated *L. fermentum* NCIM 2156 *and L. plantarum* NCIM 2083 to temperatures from 90^0C to 130^0C was studied using distilled water as a suspending medium in oil bath. 1g of microcapsules (10^{10} cells per g) was transferred in test tube containing 9 ml distilled water. After the heat treatment the content was cooled to room temperature and viable cells were enumerated.

4.5 Preparation of orange peel jelly

Sweet orange peels were collected from NIT Rourkela hostel during December 2014 and January 2015.Time and temperature required for extraction of pectin was optimized by measuring total soluble solids (6%) using hand refractometer. Jelly was made by adding equal amount of sugar to that of extract. 1 g of cells was incorporated in the orange peel jelly and the probiotic jelly was stored at room temperature for 1 week. After 1 week the probiotic jelly was dissolved in saline. From the dissolved solution, 100 µL was spreaded on MRS agar by spread plate method and the cells were enumerated after incubation at 37^0C for 48 hrs.

4.6 Viability of free cells stored in orange peel jelly

The jelly was prepared from citrus peel extract by adding equal amount of sugar i.e to increase TSS from 3% to 80%. When it is about to solidify (approx. 70^0C) 1 ml of culture of *L. fermentum* NCIM 2156 and *L. plantarum* NCIM 2083 was added separately to 20 g of jelly. This probiotic jelly was stored at room temperature for 1 week and the viability of cells was enumerated after 1 week.

4.7 Preparation of mushroom extract

Mushroom (*Pleurotus ostreatus*) was collected from Mushroom centre, Sector-1, Rourkela. The mushrooms were dried in hot air oven for two days at 70^0C. The Mushroom extract was prepared by boiling 20 g dried mushrooms in 100 ml boiling water. The extract was concentrated by evaporation.

4.8 Preparation of synbiotic microcapsules

2:1ratio of *Pleurotus ostreatus* extract and sodium alginate solution was taken to make the synbiotic microcapsules. 30 ml sodium alginate was added to the 60 ml mushroom extract then added drop wise to the $CaCl_2$ solution with help of syringe to make the synbiotic microcapsules. These microcapsules were separated by the sieve and dried. These dried synbiotic microcapsules are stored in health mix (Horlicks).

4.9 Viability of encapsulated cells stored in food matrix

1g of synbiotic microcapsules was incorporated in the health mix (Horlicks) and their survivability in food matrix was studied after2weeks.

5. Results and Discussion

5.1 Encapsulation of bacterial cells using sodium alginate beads

By using 3% sodium alginate and 0.5 M $CaCl_2$ solution, beads were prepared for *L. fermentum* NCIM 2156 and *L. plantarum* NCIM 2083. The beads are of same size, same diameter and round in nature.

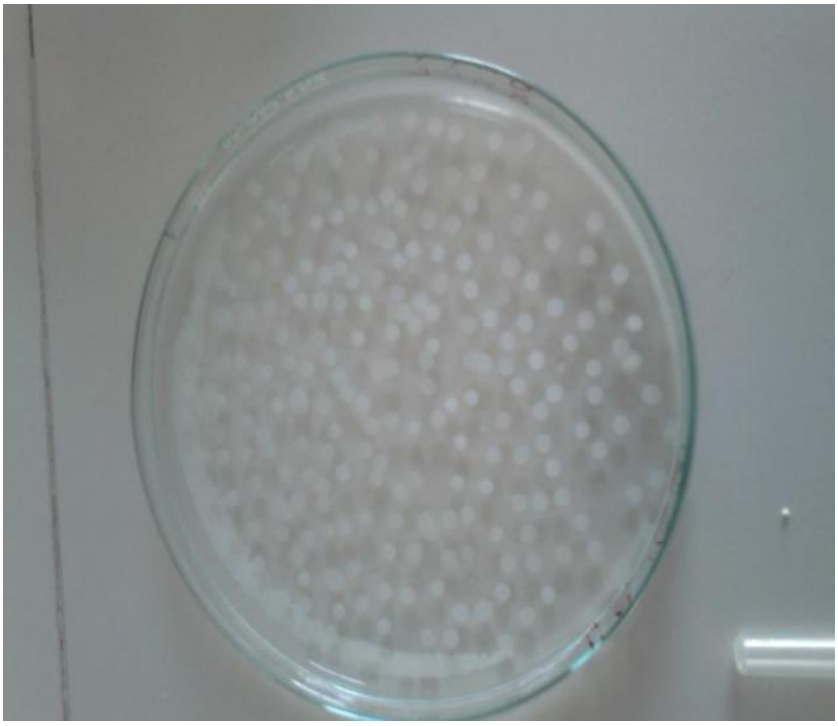


Figure 1: Encapsulated bacterial cells

5.2 Effect of temperature on viability of free cells

1 ml culture was transferred to each test tubes containing 9 ml distilled water. These test tubes were exposed to different temperatures (60^0C, 70^0C, 80^0C, and 90^0C) for different time intervals (1 min, 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min) in water bath. The cells were spreaded on MRS agar by spread plating method and their survivability was examined after incubation at 37^0C for 48 hrs. It was found that the *L. fermentum* NCIM 2156 was surviving up to 90^0C for 1 minute and *L. plantarum* NCIM 2083 was surviving up to 90^0C for 5 minute as a free cells. Hence *L. plantarum* NCIM 2083 was exposed at 100^0C for different time intervals (30 sec, 1 min, 5 min and 10 min) and studied that *L. plantarum* NCIM 2083 was surviving for 30 sec at 100^0C. The reason behind the survival of the *L. plantarum* NCIM 2083 up to 100^0C may be due to HSPs proteins or chaperons which protect proteins from denaturation as well as various cellular mechanisms such as DNA replication, RNA splicing etc, thus helping them to survive up to 100^0C as free cells.

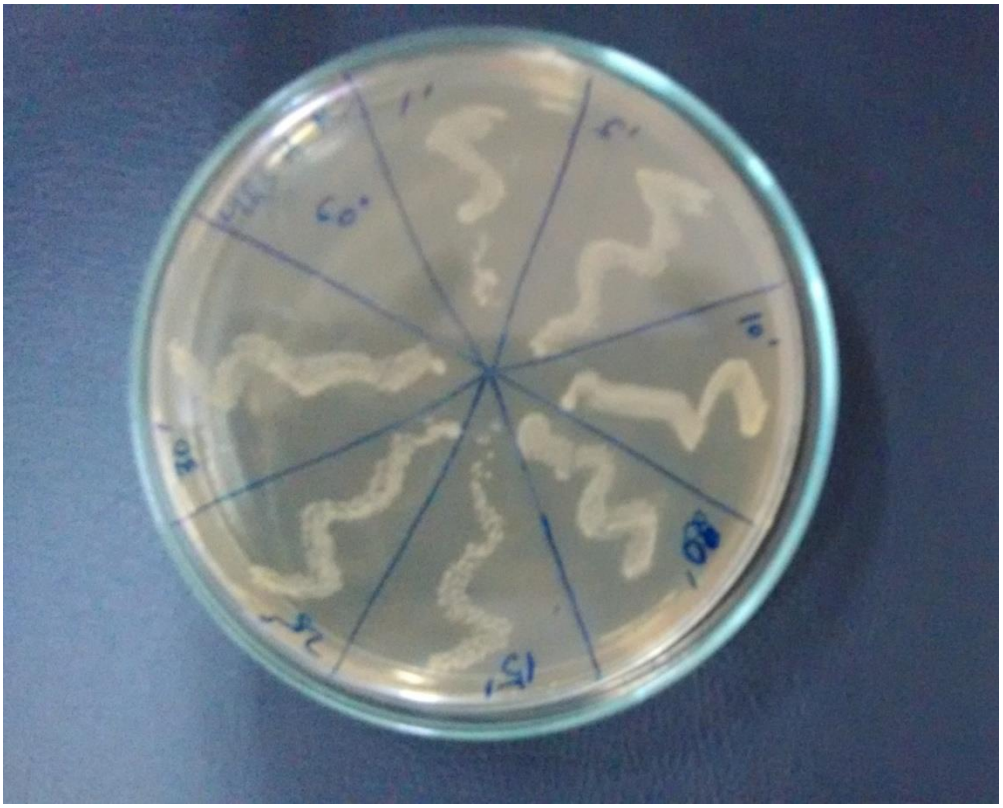


Figure 2: Plate showing surviving cells of *L. plantarum* NCIM 2083 after exposed to 60^0C for different time intervals

Table 1: Effect of temperature on survivability of free bacterial cells at 60^oC

	Time in minutes						
Name	**1**	**5**	**10**	**15**	**20**	**25**	**30**
L. fermentum NCIM 2156	S	S	S	S	S	S	S
L. plantarum NCIM 2083	S	S	S	S	S	S	S

S - Survive; NS - Not Surviving

Table 2: Effect of temperature on survivability of free bacterial cells at 70^oC

Name	**Time in minutes**						
	1	**5**	**10**	**15**	**20**	**25**	**30**
L. fermentum NCIM 2156	S	S	S	S	S	S	S
L. plantarum NCIM 2083	S	S	S	S	S	S	S

S – Survive

Table 3: Effect of temperature on survivability of free bacterial cells at 80^oC

Name	Time in minutes		
	1	**5**	**10**
L. fermentum NCIM 2156	S	S	NS
L. plantarum NCIM 2083	S	S	S

S - Survive; NS - Not Surviving

Table 4: Effect of temperature on survivability of free bacterial cells at 90^{o}C

Name	Time in minutes		
	1	**5**	**10**
L. fermentum NCIM 2156	S	NS	NS
L. plantarum NCIM 2083	S	S	S

S - Survive; NS - Not Surviving

Table 5: Effect of temperature on survivability of free bacterial cells at 100^{o}C

Name	Times			
	30s	**1min**	**5min**	**10 min**
L. plantarum NCIM 2083	S	NS	NS	NS

S - Survive; NS - Not Surviving

5.3 Effect of temperature on viability of encapsulated bacterial cells

Tolerance of encapsulated *Lactobacillus fermentum* NCIM 2156 and *Lactobacillus plantarum* NCIM 2083 to heat treatment (>90^{0}C) was studied. 1 g of microcapsules (10^{10} cells per g) were transferred in test tube containing 9 ml distilled water as a suspending medium in oil bath. After the heat treatment the content was cooled to room temperature and viable cells were enumerated. It was observed that cells surviving at a temperature >100^{0}C were exposed to same temperature for different time intervals. The strain (*Lactobacillus plantarum* NCIM 2083) which is surviving up to higher temperature as free cells was selected for encapsulation and was found that encapsulated cells was surviving up to 130^{0}C for 2 min, showing increase in survivability after encapsulation. Since encapsulation provides an extra covering which reduces the transfer of heat as well as water into the bacterial cells as a result the temperature tolerance of bacterial cells was significantly increased.

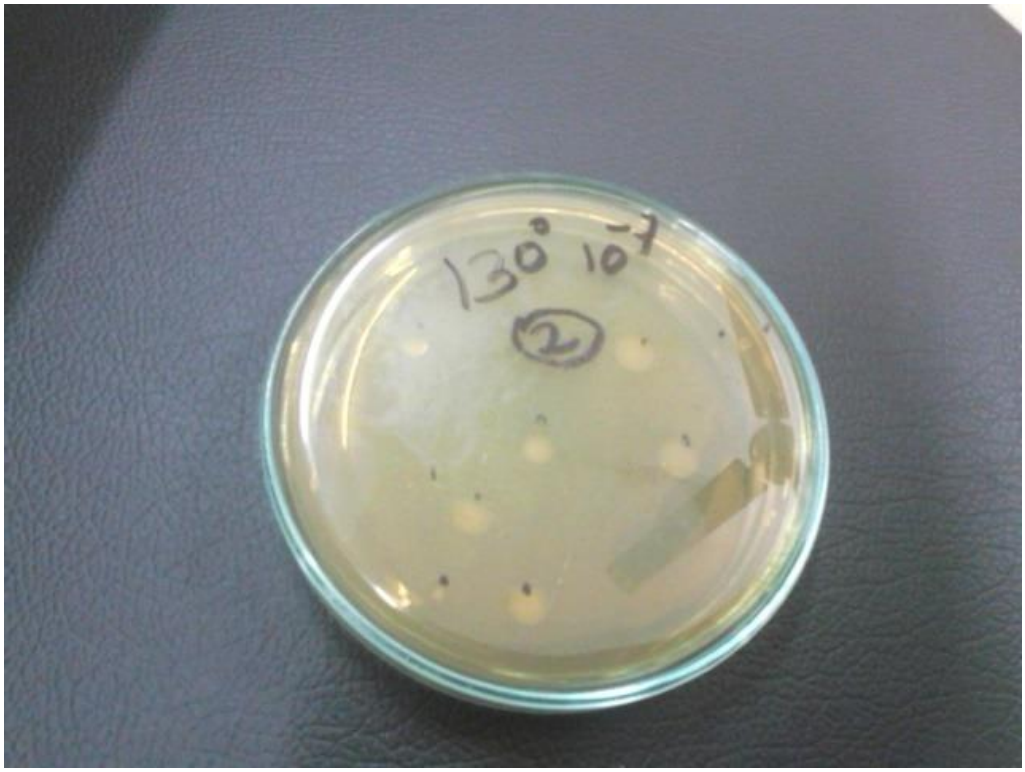


Figure 3: Plate showing surviving cells from encapsulated *L. plantarum* NCIM 2083 after exposing to 130^0C for one minute

Table 6: Effect of encapsulation on temperature tolerance of bacteria at 100°C in different time periods

Name	Time in minutes		
	5	10	15
	Control		
L. plantarum NCIM 2083	1.83	5.54	3	NS

S - Survive; NS - Not Surviving; Control - encapsulated bacteria without heat treatment

Table 7: Effect of encapsulation on temperature tolerance of bacteria at >100°C for 1min

Name	Temperatures					
	control	110°C	120°C	130°C	140°C	150°C
L. plantarum NCIM2083	4	3.9	6.2	6.7	NS	NS

Table 8: Effect of encapsulation on temperature tolerance of bacteria at 130°C in different time period

Name	Time in minutes				
	control	1	2	3	4	5

L. plantarum NCIM2083	7.0	6.7	4.1	NS	NS	NS

S - Survive; NS - Not Surviving; Control - encapsulated bacteria without heat treatment

5.4 Jelly preparation using orange peel extract

Jelly was prepared using water extract of orange peels collected from NIT Rourkela hostel during December 2014 and January 2015. Time and temperature required for extraction of pectin was optimized by measuring total soluble solids (6%) using hand refractometer. Jelly was made by adding equal amount of sugar to that of extract. Sensory analysis of the jelly with bacterial culture was done using 9 point hedonic scale.

Table 9.Sensory analysis of freshly prepared probiotic jelly added with bacteria

Sensory attributes selected for the study	Responses
Texture	Like slightly
Colour	Like very much
Flavour	Like moderately
Taste	Like slightly
After Taste	Dislike slightly
Over-all likings	Like slightly

Figure 4: Preparation of jelly

Table 10. Effect of different temperatures for 10 mins on extraction of total soluble solid (TSS) from fresh and dried peels.

Temperature(^{O}C) and Time(Min)	% of TSS (Total Soluble Solid)	
	Fresh Peels	**Dried Peel**
70	0.2	3
100	0.2	3
170	0.2	3
200	0.2	4
240	0.3	4

5.5 Viability of free cells stored in orange peel jelly

The jelly was prepared from citrus peel extract by adding equal amount of sugar i.e to increase TSS from 3% to 80%. When it is about to solidify (approx. 70°C) 1 ml of culture of *L. fermentum* NCIM 2156 and *L. plantarum* NCIM 2083 was added separately to 20 g of jelly. This probiotic jelly was stored at room temperature for a week and the viability of cells was enumerated after a week. It was found that the viability of cells was reduced to a greater extent which may be due to the higher amount of sugar added during jelly preparation which may have lead to exosmosis.

Table 11.Survival of bacterial cells during storage period of 1week

NCIM Strain	Log_{10} Number of viable cells in 20g jelly	
	Fresh	**Stored**
L. fermentum	8.8	3
L. plantarum	7.5	3.4

5.6 Viability of encapsulated cells stored in food matrix

1 g of synbiotic microcapsules was incorporated in the dry health mix (Horlicks) and their survivability in food matrix was studied after 2 week. The results shows that the cells in microcapsules without prebiotic are showing less survivability than the microcapsules with prebiotic (synbiotic) because prebiotic act as a food for the probiotics. Thus, increasing the survivability rate by 15 -16 % more than the cells without microcapsules.

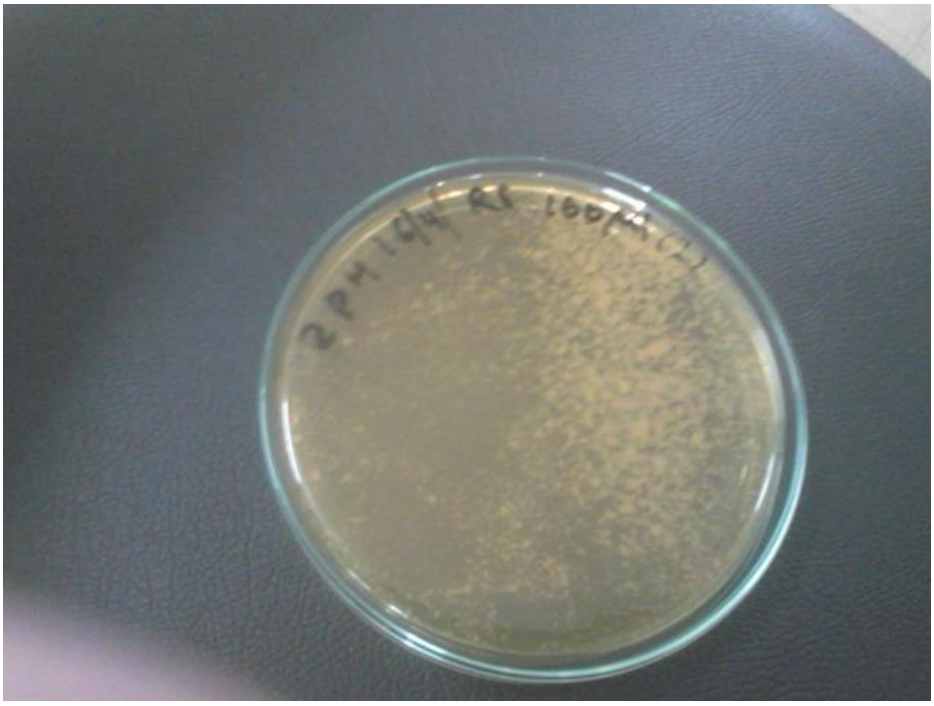


Figure 5. Number of surviving cells of *L. plantarum* NCIM 2083 in health mix (Horlicks) for 2 week.

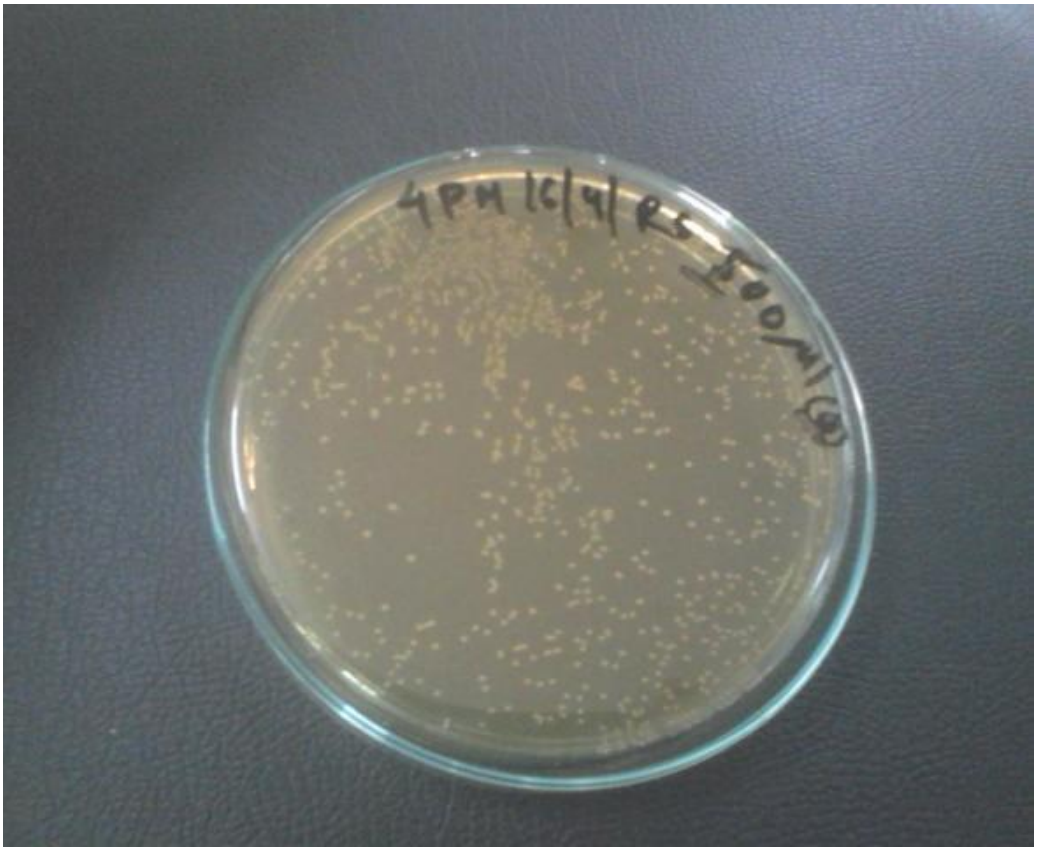
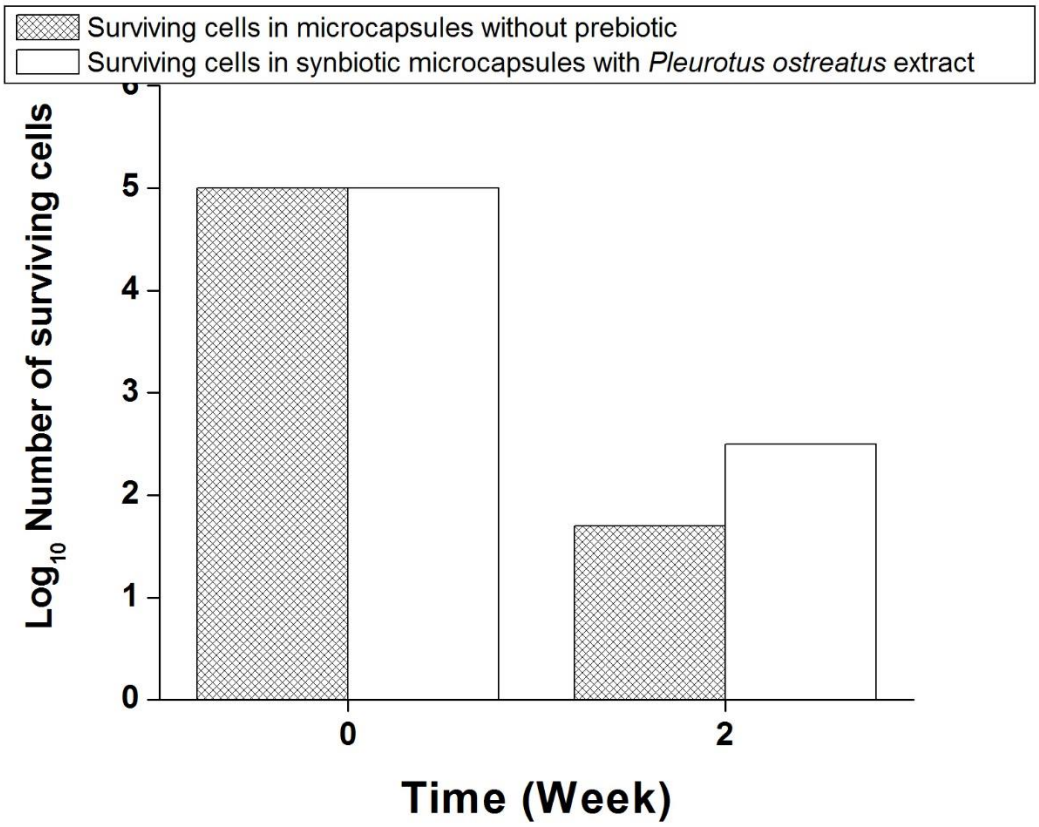
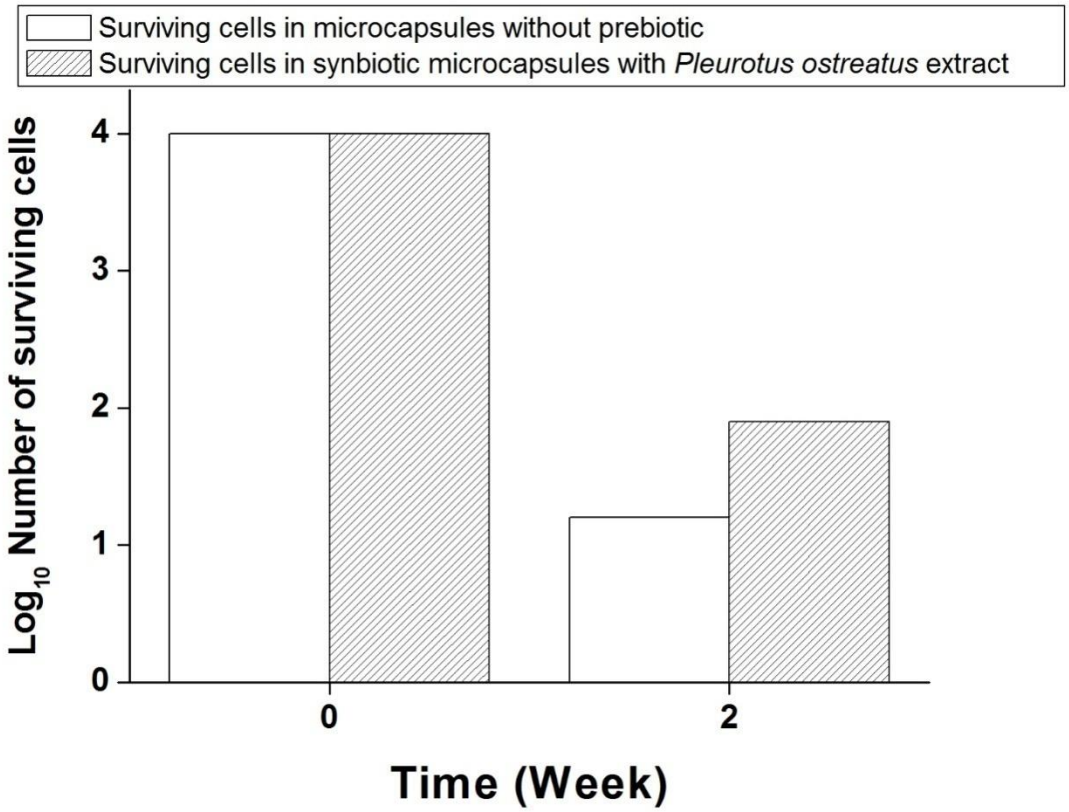


Figure 6.Number of surviving cells of *L. acidophilus* NCIM 2660 in health mix (Horlicks) for 2 week.

Graph 1: Effect of storage time on the survivability of *L. plantarum* NCIM 2083 in synbiotic microcapsules and microcapsules without prebiotic stored in health mix.



Graph 2: Effect of storage time on the survivability of *L. acidophilus* NCIM 2660 in synbiotic microcapsules and microcapsules without prebiotic stored in health mix.



6. Conclusion

The result of this study showed that the strain *L. plantarum* NCIM 2083 was found to have better stress tolerance against temperature than *L. fermentum* NCIM 2156. Hence it is the better candidate to be used in functional food such as health mix. Utilization of water extract of *Pleurotus osteratus* (mushroom) as prebiotic substance in synbiotic microencapsulation of *L. plantarum* enhanced the survivability by 17.5 % than the control when it is stored in health mix. The polysaccharides present in the mushroom extract act as a prebiotic which retards the death of the probiotics, hence helping the cells to survive for a longer period of time.

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